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# ***U.S. PATENT APPLICATION***

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***Invention:*** PHARMACEUTICAL COMPOSITION CONTAINING AN ACTIVIN OR  
INHIBIN STIMULATOR

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## ***SPECIFICATION***

## PHARMACEUTICAL COMPOSITION CONTAINING AN ACTIVIN OR INHIBIN STIMULATOR

The present invention concerns pharmaceutical preparations for promoting the healing of wounds or fibrotic disorders, in particular for promoting the healing of wounds or fibrotic disorders with reduced scarring, and for promoting the healing of chronic wounds.

By "wounds or fibrotic disorders" is meant any condition which may result in the formation of scar tissue. In particular, this includes the healing of skin wounds, the repair of tendon damage, the healing of crush injuries, the healing of eye wounds, including wounds to the cornea, the healing of central nervous system (CNS) injuries, conditions which result in the formation of scar tissue in the CNS, scar tissue formation resulting from strokes, and tissue adhesion, for example, as a result of injury or surgery (this may apply to e.g. tendon healing and abdominal strictures and adhesions). Examples of fibrotic disorders include pulmonary fibrosis, glomerulonephritis, cirrhosis of the liver, systemic sclerosis, scleroderma, proliferative vitreoretinopathy, repair following myocardial infarction, including myocardial hibernation.

In particular, there is a lack of compositions for promoting the healing of wounds or fibrotic disorders with reduced scarring. Scar tissue formation, although providing mechanical strength to a healed wound, can be unsightly and may impair the function of the tissue.

This is particularly the case in wounds which result in scar tissue formation in the CNS, the scar tissue inhibiting the reconnection of severed or re-growing nerve ends, so significantly affecting their function.

There is also a lack of compositions for use in the treatment of chronic wounds, for example venous ulcers, diabetic ulcers and bed sores (decubitus ulcers), especially in the elderly and wheel chair bound patients. Such compositions may be extremely useful in patients where wound healing is either slow or in whom the wound healing process has not yet started. Such compositions may be used to "kick-start" wound healing and may then be used in combination with compositions (e.g. those of PCT/GB93/00586) which promote the healing of wounds or fibrotic disorders with reduced scarring. Hence not only may a chronic wound be healed, but it may be healed with reduced scarring.

According to the present invention there is provided a stimulator of Activin and/or Inhibin for use in promoting the healing of wounds and fibrotic disorders with reduced scarring.

By 'stimulator' is meant anything which may stimulate the quantity or efficacy of active Activin and/or active Inhibin at a site. This may be Activin or Inhibin itself (or a pharmaceutically acceptable salt thereof) or a fragment or a partially modified form thereof. Partial modification may for example be by way of addition, deletion or substitution of amino acid residues. A substitution may for example be a conserved substitution. Partially modified molecules may, for example, have a longer half-life than their parent molecule, or they may have a different binding affinity for their receptors. A fragment may comprise at least that part of Activin or Inhibin which is required to allow it to stimulate its receptors. Alternatively, a stimulator may, for example, be an inhibitor of Activin metabolism, or it may be a stimulator of Activin synthesis, or it may be a bioprecursor of activin or inhibin. For example, it may be an analogue of a fragment of activin or inhibin which is bound by a degradative enzyme, for example a mimotope. (Geysen, H.M. *et al.*, 1987, *Journal of Immunological Methods*, 102:259-274) made to a fragment of Activin or Inhibin which is bound by an enzyme which degrades it. Such a mimotope can bind to the receptor site of the enzyme, competitively inhibiting the

binding of Activin or Inhibin (as appropriate) to the enzyme and thereby inhibiting its degradation.

It may be an antagonist of an antagonist of Activin or Inhibin. For example, it may be an antagonist of Follistatin.

Activin is a member of the TGF $\beta$  superfamily, and like the other members of this family, activins are dimeric proteins, composed of disulphide linked beta A or beta B subunits. Three different forms of Activin have been identified *in vivo*: Activin A (beta a, beta a), Activin B (beta b, beta b) and Activin AB (beta a, beta b). Herein, by "Activin" is meant all possible forms of activin. Inhibins are heterodimers of beta a or beta b chains together with a common alpha chain and are called Inhibin A (alpha beta a) and Inhibin B (alpha beta b). Herein, by "Inhibin" is meant all possible forms of inhibin (Massague, J., 1990, "The Transforming Growth Factor Beta Family", Annual Review of Cellular Biochemistry, 6: 587-641. Vale, W. *et al.*, 1990, "The Inhibin /Activin Family of Hormones and Growth Factors" in Peptide Growth Factors and Their Receptors, Volume II, M.B. Sporn and A.B. Roberts (eds), Springer-Verlag, pages 211-248).

The biological response to Activins or Inhibins is transduced by receptors which exist as heteromeric complexes of type 1 receptors (called Activin receptor like kinases (Alk) 2 and 4) and type 2 receptors which are transmembrane serine threonine kinases (Matthews, L.S. and Vale, W.W., 1993, "Molecular and Functional Characterisation of Activin Receptors", Receptor Volume 3, pages 173-181). Follistatin is an Activin binding protein which acts as an Activin antagonist *in vitro*, but *in vivo* may present Activins to their receptors (Michael, U. *et al.*, 1993, "Follistatins: more than follicle stimulating hormone suppressing proteins", Molecular and Cellular Endocrinology, Volume 91, pages 1-11).

Activin increases the number of gonadotrophs in the pituitary and causes differentiation of ovarian granulosa cells (May, K.E., 1994, "Inhibin and Activin: Molecular Aspects of Regulation and Function", *TEM* 5: 407-415). Activin A also enhances the differentiation of neuronal cells (Schubert, D. *et al.*, 1990, "Activin is a nerve cell survival molecule", *Nature*, 344: 868-870), stimulates differentiation of megakaryocytes and erythroid cells (Nishimura, M. *et al.*, 1991, "Effect of erythroid differentiation factor on megakaryocytic differentiation of L8057, a murine megakaryoblastic leukaemia cell line", *Biochem Biophysics Research Communication*, 181: 1042-1047) and induces mesoderm formation during early *Xenopus* development (Smith, J.C. *et al.*, 1990, "Identification of a potent *Xenopus* mesoderm inducing factor as a homologue of Activin A", *Nature*, 345: 729-731).

Targeted disruption of the Activin beta A chain resulted in mice with craniofacial defects which died within 24 hours after birth (Matzuk, M.M. *et al.*, 1995, "Functional analysis of activins during mammalian development", *Nature*, 274: 354-356). These mice also lacked whiskers and had abnormal whisker follicles. Activin beta A chain has been detected in the mesenchyme of developing hair follicles and embryonic skin, but not new born or adult skin (Roberts, V.J. *et al.*, 1991, "Expression of Inhibin/Activin sub-unit messenger ribonucleic acids during rat embryogenesis", *Endocrinology* 128: 3122-3129; Roberts, V.J. and Barth, S.L., 1994, "Expression of messenger ribonucleic acids encoding the Inhibin/Activin system during mid and late gestation rat embryogenesis", *Endocrinology*, 134: 914-923), in addition to the activin receptors Alk2 and Alk4 (Verschuere, K. *et al.*, 1995, "Expression of type 1 and type 1B receptors for activin in mid-gestation mouse embryos suggests distinct functions in organogenesis", *Mechanisms of Development*, 52: 109-123). Disruption of the activin-binding protein, follistatin, in transgenic mice results in abnormal whisker development and hyperkeratotic skin (Matzuk, M.M. *et al.*, 1995, "Multiple defects and perinatal death in mice deficient in follistatin", *Nature*, 374: 360-363). Disruption of the gene for the Activin/Inhibin beta B subunit resulted in subtle defects to eyelid development

(Vassail, A. *et al.*, 1994, "Activin/Inhibin beta b subunit chain disruption leads to defects in eyelid development and female reproduction", *Genes and Development*, **8**: 414-427), whilst targeted disruption of the Inhibin alpha chain caused tumour formation in the gonads (Matzuk, M.M. *et al.*, 1992, "Inhibin is a tumour suppressor gene with gonadal specificity in mice", *Nature*, **360**: 313-319).

There have been no reports of the role of either Activin, Inhibin or follistatin during wound healing, scarring or fibrosis.

However, the present inventor has found that Activin and Inhibin in fact play roles in wound healing as non-fibrotic growth factors. High levels of expression of Activin and of Activin and Inhibin receptors have been found post-wounding at wound sites, similar to TGF- $\beta_3$  (see PCT/GB93/00586). This observation is particularly surprising in light of the prior belief that Activin and Inhibin are predominantly reproductive /erythroid /neurological /mesoderm inducing factors.

Activin and Inhibin have been found to be structurally similar to TGF- $\beta_3$ , the similarity being greater than that with TGF- $\beta_1$  and TGF- $\beta_2$ . It appears that Activin and Inhibin may in fact bind to receptors similar to those bound by TGF- $\beta_3$  and as such mediate the control of scarring *via* that route.

It has also been found that the Act 2a receptor, which is bound by Activin and which is believed to be bound by TGF- $\beta_3$ , is upregulated in wound healing, especially on day 7 post-wounding. Table 1 details further the binding of the isoforms of the TGF- $\beta$  receptor family.

Hence Activin and Inhibin have similar anti-scarring properties to those of TGF- $\beta_3$  and as such Activin and Inhibin may be used to similar effect (see, for example, PCT/GB93/00586).

The stimulator may be used in conjunction with a pharmaceutically acceptable carrier, diluent or excipient. It may be used in conjunction with a pharmaceutically acceptable carrier, diluent or excipient in the preparation of a medicament for promoting the healing of wounds and fibrotic disorders with reduced scarring. Accordingly, the present invention also provides the use of a stimulator of activin and/or inhibin in the preparation of a medicament for use in promoting the healing of wounds and fibrotic disorders with reduced scarring.

Two or more stimulators according to the present invention may of course be included in a single composition or medicament or used in a single treatment.

Pharmaceutically acceptable carriers, diluents and excipients are well known - see for example Remington's Pharmaceutical Sciences and US Pharmacopeia (1984) Mack Publishing Company, Easton, PA.

Pharmaceutically acceptable carriers may for example comprise a neutral sterile cream, gel or powder for topical application, or a sterile solution for injection, irrigation or inhalation or an aerosol, or may comprise a sterile dressing for topically covering a wound or may be in the form of a tablet or capsule for enteral administration, or the carrier may comprise a biopolymer patch or a slow release device for implantation.

Stimulators of activin and/or inhibin and medicaments manufactured or prepared according to the present invention may be in the form of a composition for topical administration as a cream, gel, powder or dressing; in a solution for injection, irrigation or inhalation or aerosol, or in the form of a tablet or capsule for enteral administration. They may also comprise a biodegradable polymer forming a patch, or an implantable control release device, useful in surgical operations having a large initial release followed by a slower release later. It will be appreciated that this list is not

exhaustive, many other types of compositions being possible, such as might readily occur to one skilled in the art.

Other forms of the present invention in which are included a stimulator of activin and/or inhibin also include bandages; biocompatible, biodegradable, non-inflammatory delivery vehicles such as hyaluronic acid; implants; intradermal injections; systemic therapy for e.g. fibrosis or severe trauma or burns, for example by intraperitoneal, intravenous or oral administration; eye drops for corneal wounds or scarring; films and barriers for treating adhesions.

Application for compositions and agents for promoting the healing of wounds and fibrotic disorders with reduced scarring are well known (see for example PCT/GB93/00586, PCT/GB92/00570 and US 5,520,926) and the present invention incorporates them accordingly.

The stimulator may be used in conjunction with a composition for promoting the healing of wounds or fibrotic disorders with reduced scarring.

The stimulator may be used in conjunction with a composition for promoting the healing of chronic wounds.

Also provided according to the present invention is a method for promoting the healing of wounds or fibrotic disorders with reduced scarring comprising stimulating Activin and/or Inhibin.

The stimulation may be achieved by administering to a site activin and/or inhibin itself or a stimulator of Activin and/or Inhibin. By 'site' is meant a site of wounding or fibrotic disorder. The stimulator may be a stimulator according to the present invention. It may, for example, be an antagonist of Follistatin.

Activin and/or Inhibin may be stimulated immediately prior to wounding. It may be preferably stimulated immediately after wounding. It may be stimulated within 14 days of wounding, preferably within 7 days of wounding, more preferably within 3 days of wounding.

The method may be for use in conjunction with a method for promoting the healing of wounds or fibrotic disorders with reduced scarring.

The method may be for use in conjunction with a method for promoting the healing of chronic wounds.

The invention will be further apparent from the following description which show, by way of example only, forms of promotion of the healing of wounds and fibrotic disorders with reduced scarring.

## EXPERIMENTAL

Initial studies were undertaken to determine the expression profile of Activin in wounded tissue, relative to control tissue. These resulted in the conclusion that exogenous addition of Activin, or its related molecule Inhibin (which binds to similar receptors to Activin) or antagonism of the binding protein of Activin (Follistatin) could have anti-scarring activity. This was then tested in two sets of experiments, the first involving the use of Activin A and the second using Inhibin. The conclusions of the experiments were that Activin and Inhibin have an anti-scarring effect.

### Experiment 1

#### *Wounding*

Adult male CD1 mice were anaesthetized using halothane nitrous oxide and oxygen. Four wounds were placed on each animal, approximately one centimetre from the mid line, 20 and 40 centimetres from the base of the skull respectively. The wounds were 1 centimetre in length down to and through the panniculus carnosus. Animals were killed and wounds recovered on days 1, 3, 7, 14, 28, 60 and 80, post wounding. At least 4 wounds from 4 separate animals were analysed for each experiment. Wounds were excised, fixed in paraformaldehyde, dehydrated and embedded in wax in preparation for *in situ* hybridisation (under RNAase free conditions), or frozen in OCT (Miles Scientific), cryosectioned and utilized for immunocytochemistry.

For *in situ* hybridisation, antisense riboprobes were constructed against the Act 2a receptor, Act R1 (Alk 2) and Act R1B (Alk 4).

For immunocytochemistry, a primary antibody recognising Activin was used and detected using streptavidin biotin amplification using an FITC (fluorescein isothiocyanate) labelled secondary antibody.

As controls, non wounded adult and fetal E16 (embryonic day 16) skin were used.

### *Results*

On days 3 and 7 post wounding, enhanced staining for Activin was detected in the wound site, predominantly in fibroblasts of the wound margin and granulation tissue. Staining had returned to near normal levels by 14 days post wounding. As the antibody predominantly recognises the Activin beta A chain, it is assumed that this is the predominant isoform in the granulation tissue.

The messenger RNA for the Act 2a receptor was up-regulated in the wound margin and granulation tissue on seven days post wounding. The Alk 2 (Act R1) receptor was expressed in the mesenchyme of normal skin, but no significant elevation was detected in the wound edge or granulation tissue. By contrast, Act R1B (Alk 4) receptor was present at a much lower level in the normal skin dermis but was up-regulated in the dermal wound margin and granulation tissue of the wounds, particularly on days 7 and 14, post wounding.

In normal adult mouse skin, Alk 2 and Alk4 were expressed predominantly in the dermis and epidermis, respectively. Staining for Activin in the normal adult skin was at a marked low level in the dermis. However, fetal skin from embryonic day 16 mice showed marked staining for activin, particularly in the fetal dermis.

These staining patterns suggest that Activin and its receptors are present in fetal skin and reinduced during wound healing in adult skin. As fetal wounds heal without scarring at embryonic day 16 (Whitby, D.J. and Ferguson, M.W.J., "The extracellular matrix of lip wounds in fetal, neonatal and adult mice", *Development*, **112**: 651-668, 1991) and with reduced levels of inflammation, and hence TGF $\beta$ 1 and TGF $\beta$ 2, but enhanced endogenous dermal levels of TGF $\beta$ 3 (Whitby, D.J. and Ferguson, M.W.J., 1991, "Immunohistochemical localisation of growth factors and fetal wound healing",

Developmental Biology, 147: 207-215), it might reasonably be assumed that Activin plays a role in this scarless fetal wound healing. Hence, exogenous addition of Activin, or its related molecule Inhibin (which binds to similar receptors to Activin) or antagonism of the binding protein of Activin (Follistatin) could have anti-scarring activity.

In order to test this, the following experiment was undertaken:

### Experiment 2

#### *Materials and Methods*

Recombinant bovine Activin A (4 µg) was obtained from Innogenetics, Belgium (Cat. No. CY-035). Activin A was prepared by initially reconstituting the lyophilised powder in sterile phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and then diluting with PBS/BSA to give three doses: 100 ng/ml; 50 ng/ml; and 25 ng/ml.

Twelve adult male Sprague-Dawley rats, age- and weight-matched (220g - 250g), were anaesthetised using a mixture of equal parts halothane, nitrous oxide and oxygen. The dorsal surfaces were shaved and swabbed with 70% alcohol. Four 1 cm linear full thickness (down to and including the panniculus carnosus) incisions were made at defined anatomical positions 5 cm and 8 cm from the base of the skull, and 1cm each side of the midline.

Of the four wounds per animal, two were treated with a 100 µl dose of Activin A, one with 100 µl of PBS, and the other remained unmanipulated. All injections were intradermal, approximately 50 µl delivered down each side of the incision as close as possible to the wound without rupturing it, and were administered once daily for three days, starting immediately prior to wounding (Day 0).

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The twelve animals were divided into three groups according to the dose administered. Four animals received daily 100 µl injections of 100 ng/ml Activin A (i.e. 10 ng/100 µl injection), four received 50 ng/ml (i.e. 5 ng/100 µl injection) and the remaining four were treated with 25 ng/ml (i.e. 2.5 ng/100 µl injection). The wounds were uncovered and unsutured. Six animals, two from each treatment group, were killed 7 days pw (post-wounding) and the remaining six killed at 80 days post-wounding, all by chloroform overdose followed by dislocation of the neck. A PC based image capture system was used to save macroscopic images of the intact, shaved skin. The dorsal skin was removed and the full thickness wounds excised with a margin of approximately 0.5 cm of normal skin around the wound. One half of the tissue was fixed in formal saline and processed for routine wax histology and the other half immersed in OCT embedding medium and snap frozen over liquid nitrogen for immunocytochemical analysis.

#### *Wax histology*

7 µm sections were cut on a standard microtome and the sections stained with Haematoxylin & Eosin to examine cellularity and angiogenesis, and Masson's Trichrome stain for collagen organisation.

#### *Results*

##### *Macroscopic*

A visual analogue scoring system was devised which ranged from 0 representing normal, unwounded skin, to 10 representing hypertrophic scarring. A 10 cm unmarked line was drawn on a blank piece of paper and the four scars on the dorsal surface of each freshly killed rat were scored by placing a mark along the line between 0 and 10, with a separate line for each scar. Only the 80 day scars were scored (i.e. 6 rats).

The macroscopic appearances of the wounds treated with Activin A were very good. The scars were quite variable but the lowest doses produced the best quality macroscopic scars when compared to the controls.

*Microscopic**10 ng/100 µl injection:*

At 7 days pw, the wounds were re-epithelialised and the epithelium had flattened out, similar to unwounded epithelium. One consistent observation at 7 days post-wounding (pw) was that there were not many inflammatory cells at the top of the wound, but quite a lot at the base. The control wounds (unmanipulated and PBS treated) were also re-epithelialised but had more inflammatory cells distributed throughout the wound.

At 80 days pw, the microscopic appearance of the scars was very good. Another visual analogue scoring system was used, ranging from 0 representing normal skin to 10 representing hypertrophic scarring. The average scores are shown in Table 2. The average score for treated wounds was 2.65, PBS treated 3.3, and unmanipulated 3.65. The orientation of collagen in the treated wounds was more like that of normal skin, the collagen bundles being less densely packed, larger, and having a more basket-weave appearance (unwounded dermis has collagen bundles arranged in a basket-weave architecture), particularly towards the epidermis.

*5 ng/100 µl injection:*

At 7 days pw, the wounds were all re-epithelialised and quite cellular throughout and treated wounds appeared similar to control wounds. There was some variation in the treated wounds, with some being very cellular, and others not containing as many inflammatory cells.

At 80 days pw, the dermal architecture was good, averaging a score of 3.13, compared to PBS and unmanipulated control wounds which averaged 5.5 and 4.1 respectively. The collagen was more open and there were thicker bundles, again particularly at the top of the wound site near the epidermis.

*2.5 ng/100 µl injection:*

At 7 days pw, the treated wounds resembled the control wounds.

At 80 days pw, the treated wounds had a reasonable collagen architecture, and averaged a score of 5.25, compared to PBS and unmanipulated control wounds which averaged 5.45 and 4.25 respectively.

### *Conclusions*

These experiments show that the TGF- $\beta$  family member Activin A has an anti-scarring effect.

Both 5 ng/100  $\mu$ l injection and 10 ng/100  $\mu$ l injection treatment regimes showed considerable improvement in scarring relative to control wounds. The 2.5 ng/100  $\mu$ l injection treatment regime was probably too low. It is interesting that the highest dose appears to reduce the influx of inflammatory cells into the wound - an effect similar to that achieved with TGF- $\beta_3$ . The microscopic appearance of wounds at 80 days pw which had been treated with 10 ng/100  $\mu$ l injection Activin A was better than the controls, and 5 ng/100  $\mu$ l injection was also better than controls. Comparison between 10 ng/100  $\mu$ l injection and 5 ng/100  $\mu$ l injection treatments showed that the 10 ng/100  $\mu$ l injection treatment was superior but that the control wounds in these animals were also better, possibly the result of systemic effects of the high dose of Activin A. The lowest dose (2.5 ng/100  $\mu$ l) also slightly improved scarring although the microscopic results were closer to the control wounds.

The macroscopic appearance of the wounds was quite variable although the wounds treated with the middle (5 ng/100  $\mu$ l injection) and lowest (2.5 ng/100  $\mu$ l injection) doses appeared to be better than their respective controls. The wounds treated with the highest (10 ng/100  $\mu$ l injection) dose were macroscopically quite variable, but an obvious effect may have been negated by the quality of the control wounds which were on the same

animal and therefore perhaps improved by systemic effects of the high dose of Activin A.

### Experiment 3

#### *Materials and Methods*

Porcine Inhibin (20 µg per vial) was obtained from the National Institute for Biological Standards and Control, Porters Bar, UK (Cat No 86/690)

The inhibin was reconstituted in sterile PBS with 0.1% BSA to a stock solution of 20 µg/ml and further diluted to 0.1, 1 and 5 µg/ml (i.e. 10, 100 and 500 ng/100 µl injection).

#### *Pilot Experiment*

Twelve adult male Sprague-Dawley rats, age- and weight-matched (220g - 250g), were anaesthetised using a mixture of equal parts halothane, nitrous oxide and oxygen. The dorsal surfaces were shaved and swabbed with 70% alcohol. Four 1cm linear full thickness (down to and including the panniculus carnosus) incisions were made at defined anatomical positions: 5 cm and 8 cm from the base of the skull, and 1cm each side of the midline.

Two wounds per animal were treated with a 100 µl dose of Inhibin, one with 100 µl of either PBS/BSA or PBS alone, and one remained unmanipulated. All injections were intradermal. The first injection was administered at the wound site immediately prior to wounding (day 0) and then for two days following wounding. 50 µl was delivered down each side of the incision as close as possible to the wound without rupturing it.

The 12 animals were divided into three groups according to the dose administered. Four animals received daily injections of 10 ng/100 µl, four received 100 ng/100 µl and the remaining four were treated with 500 ng/100 µl. The wounds were uncovered and unsutured. Six animals, two from each treatment group, were killed 7 days post

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wounding and the remaining six killed at 80 days post-wounding, all by chloroform overdose followed by dislocation of the neck. A PC based image capture system was used to save macroscopic images of the intact, shaved skin. The dorsal skin was removed and the full thickness wounds excised with a margin of approximately 0.5 cm of normal skin around the wound. One half of the tissue was fixed in formal saline and processed for routine wax histology and the other half immersed in OCT embedding medium and snap frozen over liquid nitrogen for immunocytochemical analysis.

**Wax Histology:** 7  $\mu$ m sections were cut on a standard microtome and the sections stained with Haematoxylin & Eosin to examine cellularity and angiogenesis, and Masson's Trichrome stain for collagen organisation.

## *Results*

### *Macroscopic*

A visual analogue scoring system was devised which ranged from 0 representing normal, unwounded skin, to 10 representing hypertrophic scarring. A 10 cm unmarked line was drawn on a blank piece of paper and the four scars on the dorsal surface of each freshly killed rat were scored by placing a mark along the line between 0 and 10, with a separate line for each scar. Only the 80 day scars were scored (i.e. 6 rats, 24 wounds).

The appearances of the wounds treated with Inhibin were variable at 80 days. Of the wounds treated with the highest dose (500 ng/injection), one was an extremely good, fine linear scar and the others were similar to controls. Wounds treated with PBS/BSA were also similar to unmanipulated controls. The wounds treated with the middle dose of Inhibin were also quite similar to control wounds. Two of the wounds treated with the lowest dose had very fine linear scars, barely discernible from the surrounding unwounded dermis, while the other scars were similar in appearance to controls. Overall, the macroscopic results suggested that the lowest or highest doses of Inhibin may improve scarring.

## *Histology*

### *7 days post-wounding*

Overall, the treated wounds resembled unmanipulated or PBS treated control wounds at 7 days. The wounds contained a lot of inflammatory cells, had re-epithelialised and were variable in width. The control wounds treated with PBS/BSA were very cellular, were very wide and in one case had not re-epithelialised.

### *80 days post-wounding*

A visual analogue scoring system also ranging from 0 representing normal, unwounded skin, to 10 representing hypertrophic scarring, was used to score the histology slides at 80 days post-wounding (Table 3). Some of the wounds treated with the highest dose of Inhibin (500 ng/100 µl injection) had a good dermal architecture, the collagen bundles were thick and in a random organisation resembling the normal basket-weave pattern of unwounded dermis. In most of the other wounds the collagen was dense and in parallel alignment, resembling the control wounds. The average score was 4.49 for wounds treated with 500 ng Inhibin/100 µl injection, 5.6 for untreated control wounds and 5.08 for PBS control wounds. Wounds treated with the middle dose (100 ng/100 µl injection) were similar to PBS controls (scores were 7.58 and 7.9 respectively), the collagen fibres were thick but densely packed, mostly at the top of the wound. (The unmanipulated controls had a good score of 4.9 in this group). The collagen in the wounds treated with the lowest dose of Inhibin (10 ng/100 µl injection) was orientated in an open, random fashion but once again at the top of the wound, the collagen was quite densely packed. These wounds scored similarly to the controls (see Table 3).

## *Conclusions*

The highest dose of Inhibin appeared to have a slight anti-scarring effect, with the microscopic results correlating with macroscopic data. The PBS/BSA control appeared to produce worse scars at 80 days post-wounding and at 7 days post-wounding the

wounds contained much larger numbers of inflammatory cells. Reconstituting the Inhibin in PBS alone may have a more marked anti-scarring effect.

#### Experiment 4

##### *Follow-up Experiment*

##### *Materials and Methods*

Porcine Inhibin (20 µg per vial) was obtained from the National Institute for Biological Standards and Control, Potters Bar, UK (Cat. No. 86/690).

The Inhibin was reconstituted in sterile PBS with 0.1% BSA to a stock solution of 20 µg/ml and further diluted to 2.5, 10 and 15 µg/ml (i.e. 250, 1000 and 1500 ng/100 µl injection).

The surgical technique used was as before except for the numbers of animals used (18; n=72) and there was an extra time point at 40 days post-wounding.

Two wounds per animal were treated with a 100 µl dose of Inhibin, one with 100 µl of either PBS/BSA or PBS alone, and one remained unmanipulated. All injections were intradermal. The first injection was administered at the wound site immediately prior to wounding (day 0) and then for two days following wounding. 50 µl was delivered down each side of the incision as close as possible to the wound without rupturing it.

The 18 animals were divided into three groups according to the dose administered. Six animals received daily 100 µl injections of 250 ng, six received 1000 ng and the remaining six were treated with 1500 ng. The wounds were uncovered and unsutured. Six animals, two from each treatment group were killed 7 days post-wounding, six at 40 days post-wounding and the remaining six killed at 80 days post-wounding, all by chloroform overdose followed by dislocation of the neck. A PC based image capture

system was used to save macroscopic images of the intact, shaved skin. The dorsal skin was removed and the full thickness wounds excised with a margin of approximately 0.5 cm of normal skin around the wound. One half of the tissue was fixed in formal saline and processed for routine wax histology and the other half immersed in OCT embedding medium and snap frozen over liquid nitrogen for immunocytochemical analysis.

#### *Wax Histology*

7  $\mu\text{m}$  sections were cut on a standard microtome and the sections stained with Haematoxylin & Eosin to examine cellularity and angiogenesis, and Masson's Trichrome stain for collagen organisation.

#### *Results*

##### *Macroscopic*

The standard visual analogue scoring system was used which ranged from 0 representing normal unwounded skin, to 10 representing hypertrophic scarring. A 10 cm unmarked line was drawn on a blank piece of paper and the four scars on the shaved dorsal surface of each freshly killed rat were scored by placing a mark along the line between 0 and 10, with a separate line for each scar. Only the 40 and 80 day scars were scored (i.e. 6 rats, 24 wounds at each time point).

Macroscopic analysis at 40 days suggested that the wounds treated with the highest dose of inhibin (1500 ng/100  $\mu\text{l}$  injection had the least obvious scars (average score 4.1) compared to wounds which had been treated with 1000 ng/100  $\mu\text{l}$  injection or 250 ng/100  $\mu\text{l}$  injection (average scores of 5 and 4.6 respectively). However, at 80 days, the average macroscopic score (4.51) for the wounds treated with the lowest dose of inhibin (250 ng/100  $\mu\text{l}$  injection) was considerably better than scores for the two higher doses (1500 ng/100  $\mu\text{l}$  injection and 1000 ng/100  $\mu\text{l}$  injection), which had similar average scores of 5.275 and 5.375 respectively.

### *Histology*

#### *7 days post-wounding*

All wounds had re-epithelialised at 7 days post-wounding. There were no differences between PBS treated and unmanipulated control wounds. There were high numbers of inflammatory cells at the base of the wounds treated with 1500 ng/100  $\mu$ l injection and 1000 ng/100  $\mu$ l injection and there was not a large amount of new collagen compared to control wounds. The wounds treated with 250 ng/100  $\mu$ l injection were narrow, did not contain many inflammatory cells and had a lot of new collagen.

#### *40 and 80 days post-wounding*

The standard visual analogue scoring system was used to evaluate the 40 and 80 day wounds. The results are shown in Table 4.

At 40 days, the wounds treated with 250 ng/100  $\mu$ l inhibin injections had the worst dermal architecture, the collagen was densely packed and in parallel alignment, reflected in an average score of 7.4. The wounds treated with 1000 ng/100  $\mu$ l injection had an average score of 6.0 and were similar to PBS treated control wounds (6.2). The unmanipulated control wounds in the groups treated with 1000 and 1500 ng/100  $\mu$ l injection had the best average scores, possibly indicating a systemic effect. The histological scores for the wounds treated with 1500 ng/100  $\mu$ l injection were in agreement with the scores for macroscopic appearance, and reflected the superior dermal architecture observed at this stage (4.7).

At 80 days post-wounding, the histological appearance of the wounds treated with 250 ng/100  $\mu$ l injection was better than the wounds treated with 1000 or 1500 ng/100  $\mu$ l injection. The collagen bundles were less densely packed and in a more random organisation, particularly at the top of the scar, just below the epidermis. Only one scar treated with this dose was wide and of poor quality. The wounds which had received 1000 ng/100  $\mu$ l injection treatment had densely packed collagen throughout the wounds

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and the wounds treated with 1500 ng/100  $\mu$ l injection, although one had relatively open collagen orientation at the top, were in general very wide at the base where the collagen was particularly dense.

*Summary*

These results suggest that the lowest dose of inhibin used in this investigation (250 ng/100  $\mu$ l injection) had anti-scarring effects. The pilot experiment suggested that a dose of 500 ng/100  $\mu$ l injection also had slight anti-scarring effects. It appears therefore that exogenous addition of inhibin has an antiscarring effect and the data suggest the optimum dose of inhibin is between 250 and 500 ng/100  $\mu$ l injection in this treatment regime.

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**Table 1:** The TGF- $\beta$  Receptor family and their known affinities for TGF- $\beta_{1,2}$  and  $\beta_3$ , Activin, BMP 2,4 and MIS

Type I Receptors	TGF- $\beta$	Activin	BMP 2,4	MIS
TGF- $\beta$ RI	✓			
Act R-I $\beta$		✓		
Atr-I		✓		
BRK-I			✓	
RPK-I			✓	
Act R-I		✓		
TSR-I	✓	✓		
Brk-43E			✓	
Brk-25D			✓	
DAF-I				
Type II Receptors				
Act R-II		✓		
Act R-IIB		✓		
Atr II		✓		
TGF- $\beta$ RII	✓			
Daf 4			✓	
C14				?

BMP2,4 = Bone Morphogenetic Proteins

MIS = Mullerian Inhibiting Substance

Table 2: Average Histological Scores (80 days post-wounding) Rat Wounds  
Treated with Activin A.

Group	Dose (ng/100 µl injection)	Average Score		
		Treated Wounds	PBS	U
A	10	2.625	3.3	3.65
B	5	3.13	5.5	4.075
C	2.5	5.25	5.45	4.25

Dosages relate solely to Activin A treated wounds

PBS = PBS treated control wounds

U = Unmanipulated control wound

Table 3: Average Histological Scores (80 days post-wounding) of Rat Wounds Treated with Inhibin.

Group	Dose (ng/100 $\mu$ l injection)	Average Score			
		Treated Wounds	PBS	PBS/BSA	U
A	10	5.13	4.75	5.7	4.675
B	100	7.58	7.9	8.0	4.9
C	500	4.49	5.08		5.6

Dosages relate solely to Activin A treated wounds

PBS = PBS treated control wounds

PBS/BSA = PBS/BSA treated control wounds

U = Unmanipulated control wound

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**Table 4:** Average Histological Scores (40 days and 80 days post-wounding) of Wounds Treated with Inhibin

**Group A:**

<i>Dose</i>	<i>Average Score</i>	
	<i>40 days</i>	<i>80 days</i>
250 ng/100 µl injection	7.4	4.51
PBS	*	5.15
U	7.6	5.25

**Group B:**

<i>Dose</i>	<i>Average Score</i>	
	<i>40 days</i>	<i>80 days</i>
1000 ng/100 µl injection	6.0	5.225
PBS	6.2	-
U	3.9	-

**Group C:**

<i>Dose</i>	<i>Average Score</i>	
	<i>40 days</i>	<i>80 days</i>
1500 ng/100 µl injection	4.7	5.2
PBS	4.6	-
U	2.0	-

\* this denotes where results are not yet available.